Chapter 8

Concluding Remarks

A synthesis is given of the results presented in the previous chapters. It is focussed on the implications of these results for biosurfactant-enhanced soil remediation. In Chapter 1 it was concluded that in order to better understand the effect of (bio)surfactants on biodegradation of organic soil contaminants, more insight is needed into three topics. These topics were the effect of biosurfactants on the processes that limit the biodegradation rate of hydrophobic soil contaminants, the interaction between a biosurfactant and the biosurfactant-producing microorganism, and the factors that determine whether a strain can profit from surfactant addition. It will be discussed how the results presented in this thesis contribute to understanding these topics.

WHICH PROCESSES ARE STIMULATED BY RHAMNOLIPID?

Desorption of soil contaminants. Surfactants may stimulate the biodegradation rate of organic soil contaminants by stimulating desorption in situations where desorption of the contaminants from soil is the rate-limiting step (Chapter 1). The effect of rhamnolipid on desorption of phenanthrene from soil was described in Chapter 2. The results showed that rhamnolipid stimulates the removal of sorbed phenanthrene from soil by two processes: by solubilization and by increasing desorption rate constants. In other words, rhamnolipid influences both the equilibria and the kinetics of sorption of hydrophobic compounds to soil. Therefore, it is expected that rhamnolipid can increase the biodegradation rate of substrate sorbed to soil.

When desorption of the contaminants from soil is limiting the biodegradation rate, the extent of solubilization of the substrate by surfactant will not influence the biodegradation rate, nor will it be important whether the substrate can be directly taken up from the micellar pseudophase. The effect of surfactants on the biodegradation rate in these situations is solely determined by their stimulation of the desorption rate. The importance of the effect of surfactants on the desorption kinetics of contaminants and the relative lack of knowledge on this topic (Deitsch and Smith, 1995; Yeom et al., 1996a; Sahoo and Smith, 1997; Chapter 2) underscores the need for further research on how and to which extent surfactants can influence desorption kinetics of organic contaminants in soil.

Mass transfer of liquid soil contaminants from soil to the bulk aqueous phase. The rate of biodegradation of liquid or solid contaminants in soil may be enhanced by surfactants when surfactants enhance dissolution rates. Rhamnolipid stimulated the mass transfer of non-aqueous liquid substrate (*i.e.* non-aqueous phase liquid, NAPL) from porous matrices to the aqueous phase under the hydrodynamic conditions of the column experiments (Chapter 6). The liquid contaminant hexadecane was removed from the columns by dissolution and by detachment of microscopic droplets or mobilization. The correlation between interfacial tracer retardation, NAPL removal rates, and the specific surface areas for the different matrices as reported in Chapter 5 suggests that the mass transfer rates of hexadecane from the matrices to the mobile aqueous phase were correlated to the NAPL-water interfacial area. The removal rates of hexadecane in the presence of rhamnolipid was also correlated with the specific surface areas of the matrices (Chapter 6). This suggests that the mass transfer rate during continuous flow conditions was determined

by the hexadecane-water interfacial area, both in the absence and in the presence of rhamnolipid. In this respect, hexadecane removal was similar to a dissolution process, and not to a mobilization process. Mobilization of hexadecane by rhamnolipid may be enhanced by optimizing the pH and electrolyte concentration (Bai et al., 1998). The stimulation by rhamnolipid of mass transfer rates of hexadecane from the matrix to the aqueous phase suggest that rhamnolipid may enhance *in situ* bioremediation and soil remediation by pump and treat technology (Pennell et al., 1993; Bai et al., 1997). During *in situ* cleanup the soil is not agitated and the hydrodynamic conditions resemble the conditions in the column experiments.

Under conditions of higher agitation, rhamnolipid and other surfactants did not stimulate the mass transfer of hexadecane from silica to the bulk aqueous phase (Chapter 6). Even under conditions that were optimized for enhancing mobilization of hexadecane from sand by rhamnolipid (Bai et al., 1998), rhamnolipid did not enhance the biodegradation rate of hexadecane present in silica (Wachter, 1997). Controls showed that growth of *P. aeruginosa* UG2 on hexadecane present as a second liquid phase was not retarded under these conditions (Wachter, 1997). The absence of any stimulatory effect of rhamnolipid on biodegradation of hexadecane present in silica thus indicates that rhamnolipid does not enhance mass transfer rates of NAPLs that are present in small (*e.g.*, 6 nm) pores under conditions of high agitation (Chapter 6). However, NAPLs in soil will generally not be present in small pores, due to the by-passing by NAPLs of low-permeability areas and to the difficulty to displace the aqueous phase from these areas during contamination (Mayer and Miller, 1996). For NAPLs present in larger pores (*e.g.*, 300 nm), the biodegradation rate under conditions of high agitation was not limited by the mass transfer of the contaminant from the matrix to the bulk aqueous phase (Chapter 6) and a potential stimulation by surfactants of these mass transfer rates will not result in a faster biodegradation rate.

Facilitated transport. The rate of soil bioremediation may be limited when by a spatial separation between the contaminants and the microbial population, especially when the soil is not macroscopically well mixed as during *in situ* remediation. In those cases, the desorbed or dissolved contaminants must be transported to the bacteria (Harms, 1997; Herman et al., 1997a; Angelova and Schmauder, 1999). Rhamnolipid was able to facilitate the transport of polycyclic aromatic hydrocarbons (Chapter 2,3) and had the greatest transport-facilitating effect on the more hydrophobic contaminants (Chapter 3). This property could be important for enhancing biodegradation rates during *in situ* bioremediation (Volkering et al., 1998).

Uptake of substrate. The rate-limiting step during the biodegradation of hexadecane present as a second liquid phase by *Pseudomonas aeruginosa* was the uptake of the substrate (Chapter 6). The stimulation of the biodegradation rate by rhamnolipid therefore was expected to result from the stimulation of the uptake rate. In Chapter 7 it is shown that rhamnolipid indeed enhanced uptake of hydrophobic substrates by cells of *P. aeruginosa* UG2 and that this rhamnolipid-facilitated uptake can explain the observed stimulation of hexadecane biodegradation by rhamnolipid. In these cases, the stimulation by surfactant of the biodegradation rate depends on the fact whether (pseudo)solubilized substrate can directly be taken up. This is in contrast to situations where biodegradation is limited by desorption (or dissolution) rates.

The results presented in Chapter 6 and 7 show that the stimulation of hexadecane biodegradation by *P. aeruginosa* UG2 is specific for rhamnolipid and that rhamnolipid does not stimulate hexadecane biodegradation by other biosurfactant-producing strains to the same extent. This type of specificity has been observed previously, also for a sophorolipid biosurfactant (Hommel, 1990).

The specificity could not directly be explained by the physico-chemical properties of the surfactants (Chapter 6). It was concluded that the enhancement of the bioavailability observed during these experiments was caused by a specific stimulation by rhamnolipid of hexadecane uptake by the cells. This rhamnolipid-enhanced uptake will be discussed below.

Adsorption of rhamnolipid. Adsorption of rhamnolipid to soil can adversely affect its stimulation of biodegradation rates since adsorption results in surfactant loss and reduced mobility, and creates new adsorption sites for hydrophobic compounds. Adsorption of rhamnolipid to soil was low compared to adsorption of polycyclic aromatic hydrocarbons (Chapter 2). While biosurfactant adsorption did not have a large impact on the removal of phenanthrene from sandy soils (Chapter 2), it is expected to be of importance if rhamnolipid is applied in situations where adsorption is more pronounced. For instance, adsorbed surfactant significantly increased sorption of polycyclic aromatic hydrocarbons to humic acid coated silica (Chapter 3).

Since rhamnolipid is a multicomponent surfactant, preferential adsorption several of components of the surfactant mixture will result in changes in composition of the rhamnolipid remaining in the aqueous phase. Due to preferential adsorption of the more hydrophobic components to sandy soils, the rhamnolipid mixture that remained in the aqueous phase was enriched in the more hydrophilic components, both during column studies and batch studies (Chapter 4). This change in composition influences the physico-chemical properties of the surfactant such as the CMC (Chapter 4). Preferential adsorption may also influence the effect of rhamnolipid on biodegradation of organic compounds since different rhamnolipid components influenced the biodegradation rate to a different extent (Zhang et al., 1997).

Bioavailability enhancement. In conclusion, the stimulation by rhamnolipid of desorption and dissolution rates of hydrophobic contaminants may enhance their bioavailability, and may, therefore, enhance bioremediation rates. However, the stimulation by rhamnolipid of the uptake of substrates might be more important physiologically since it enables the largest enhancement of the growth rate and will be most efficient in terms of the amount of substrate that is made bioavailable per amount of rhamnolipid produced.

WHAT DETERMINES THE SPECIFIC STIMULATION BY RHAMNOLIPID OF HEXADECANE BIODEGRADATION BY *P. aeruginosa* UG2?

The biodegradation of hexadecane by *P. aeruginosa* UG2 is stimulated to the greatest extent by rhamnolipid, which seems to result from its specific stimulation of the uptake rates of hydrophobic substrates (Chapter 6 and 7). This is remarkable, since in contrast to specific uptake mechanisms for carbohydrates, inorganic and organic ions, vitamins, long-chain fatty acids (Black, 1991), and other compounds, no such mechanism is known for hydrophobic compounds. The necessity for such a biosurfactant-facilitated uptake mechanism would result from the impermeability of the cell envelope of Gram negative bacteria towards hydrophobic compounds (Hancock and Bell, 1988).

A biosurfactant-facilitated uptake mechanism would be fundamentally different from the established passive diffusion mechanism or 'hydrophobic pathway' that is generally assumed to govern uptake of hydrophobic compounds by gram-negative bacteria (Hancock and Bell, 1988). Since rhamnolipid does not accumulate in the cell or partition into the cellular envelope (Zhang and Miller, 1994), its interaction with the cell is short-lived and most probably restricted to the outermost surface of the cell. Furthermore, it is likely that the outer membrane lipopolysaccharide layer poses the most important barrier to uptake. Therefore, a

specific interaction seems to exist between rhamnolipid and a component either in the outer membrane or in the LPS layer of *P. aeruginosa*. This component might be a protein or an LPS constituent.

Energy-dependent transport over the outer membrane is known for siderophores (Kadner and Heller, 1995), Vitamin B12 (Kadner and Heller, 1995), bacteriocins (Traub and Braun, 1994; Rakin et al., 1996), and for a wide variety of drugs by outer membrane-located multidrug efflux pumps (Kohler et al., 1997; Zhao et al., 1998). An example of a protein that conveys energy from the (proton motive force of the) cytoplasmic membrane to the outer membrane is TonB. TonB spans the periplasmic space and transduces energy by direct contact to outer membrane receptor proteins containing a TonB box (Kadner and Heller, 1995; Cornelissen et al., 1997). TonB is also present in *P. aeruginosa* (Poole et al., 1996). The protein that serves as the receptor for the siderophore ferric enterobactin in *E. coli*, FepA, is a TonB-dependent, energy-dependent, ligand-gated porin (Jiang et al., 1997; Payne et al., 1997). The mechanism of rhamnolipid-facilitated uptake of hydrophobic compounds and of uptake of siderophore-bound iron by bacteria are similar in the sense that both involve a carrier (*i.e.* siderophore or biosurfactant) and are energy dependent (Hancock and Bell, 1988; Screen et al., 1995). At this moment one can only speculate about the mechanism of rhamnolipid-enhanced uptake of hydrophobic substrates. A further biochemical and molecular characterization of the mechanism is desirable.

WHICH STRAINS CAN PROFIT FROM SURFACTANT ADDITION?

The effect of (bio)surfactants on the biodegradation of organic compounds is highly strain-dependent. Of the strains used in Chapter 7, only *P. aeruginosa* was significantly stimulated by exogeneous addition of its own biosurfactant to degrade hexadecane. Only for this strain was the cell surface hydrophobicity lower during growth on hexadecane compared to glucose. Furthermore, the biosurfactants produced by *Acinetobacter calcoaceticus, Rhodococcus erythropolis, Arthrobacter paraffineus* were either cell bound or active when cell bound. It is suggested that *Ac. calcoaceticus, R. erythropolis, Ar. paraffineus,* and strain BCG112 take up hexadecane via attachment of the cells to hydrocarbon droplets (Chapter 7). Exogeneous addition of surfactant inhibits biodegradation by such strains, likely because surfactants render these droplets hydrophilic and thereby reduce attachment of the cells to the surface of these droplets (Stelmack et al., 1999). In general, strains with a high cell surface hydrophobicity seem to be less prone to stimulation by surfactants (Zhang and Miller, 1994; Churchill, P.F. and Churchill, 1997; Herman et al., 1997b). Bouchez-Naïtali et al. screened a set of 61 hexadecane-degrading soil bacteria for their cell hydrophobicity, interfacial and surface tensions of the culture supernatants, and production of glycolipid extracellular biosurfactants (Bouchez-Naïtali et al., 1999). These data were used as criteria to discriminate whether the strains take up substrate by attachment to the substrate or whether they take up pseudosolubilized substrate. It would have been interesting to determine whether biodegradation of hexadecane by these strains could be stimulated by surfactants and to determine whether this was correlated with the previously mentioned criteria. It seems that biodegradation by strains that take up substrate by attachment to the substrate can not be stimulated by surfactants

IMPLICATIONS FOR SOIL REMEDIATION

The work described in this thesis was aimed at determining whether biosurfactants could be effectively applied for enhancing the biodegradation of hydrophobic soil contaminants. However, several results are

also of interest for the application of biosurfactants for surfactant-enhanced removal by pump and treat. In the next paragraphs, the implications of the results described in this thesis for both types of applications will be discussed. Although most of the work was done with a rhamnolipid biosurfactant, some general conclusions will be derived.

Before biosurfactants can be applied on a wide scale for soil remediation, it must be established whether the positive effects of biosurfactants on soil quality outweigh the negative effects. Positive effects of biosurfactant addition or stimulation of biosurfactant production must include the reduction of the degree of contamination and the reduced toxicity of the rest contamination. Negative effects may include the increased leaching of contaminants and the toxicity of biosurfactants to soil fauna and flora.

Use of biosurfactants for enhancing removal of contaminants. The use of biosurfactants for enhancing removal of contaminants from soil by pump and treat is expected to be successful. Positive effects have been demonstrated in this thesis for adsorbed contaminants (Chapter 2 and 3) and for a contaminant present as a non-aqueous phase liquid (Chapter 6). For this type of applications, biosurfactants must be compared to synthetic surfactants based on cost, performance, biodegradability and toxicity. Biosurfactants are not expected to have an intrinsic advantage above synthetic surfactants for these applications.

When contaminants are to be removed from soil by biosurfactants, a contaminated waste stream has to be treated elsewhere (*e.g.*, NOBIS 91-5-09. Volkering and Noordman, 1996, 1997). The separation of removal and degradation might have several technological advantages such as the better control over both removal and biodegradation and the possibility to optimize both processes independently.

Complete removal of all contaminants from soil, either by biodegradation or by pump and treat, requires an extended period of time, even in the presence of rhamnolipid. Results from Chapter 2 indicate that application of biosurfactants for enhancing removal of contaminants is most efficient when their removal is not obstructed by desorption rates, *i.e.* when their transport is determined by equilibrium partitioning. Therefore, it is expected that the application of (bio)surfactants for enhancing the abiotic removal of contaminants from soil can be efficient when it is aimed to speed-up removal of (that fraction of) the contamination for which removal is not obstructed by slow desorption or dissolution rates. After removal of this fraction, removal of the remainder will be kinetically-constrained (Noordman et al., 1997). This rest contamination will therefore be less mobile or display less toxicity (Alexander, 1995; Noordman et al., 1997; Tiehm et al., 1997; Robertson and Alexander, 1998). Under certain circumstances the remaining contamination may be acceptable since it poses a lower risk to the environment.

Use of biosurfactants for enhancing biodegradation. The effectivity of biosurfactants for stimulating biodegradation of contaminants is uncertain given the specificity observed between biosurfactant and organism. Addition of biosurfactants will stimulate some organisms but will inhibit others. Further experiments under (simulated) field conditions must reveal whether the balance is positive or negative. Because of the specific interactions between biosurfactants and organisms, it might be beneficial to use biosurfactants produced by the indigenous population (Volkering and Noordman, 1996, 1997). It can also be argued that due to natural selection a population that can profit from surfactant addition will automatically adapt. This would suggest that addition of any surfactant will be effective provided that the surfactant enhances mass transfer rates. However, the adaptation of a bacterial community might be too slow for this scenario to be effective (Fig 1.3A). Apart

from addition of biosurfactants, their production *in situ* might be stimulated. Additional research would be required to investigate whether *in situ* biosurfactant production can be optimized.

The stimulation by rhamnolipid of biodegradation of aliphatic compounds by *P. aeruginosa* is impressive (Hitsasuka, 1971; Zhang and Miller, 1992; Chapter 6 and 7). The stimulation of the biodegradation rate by rhamnolipid is expected to be especially pronounced in situations where uptake of the substrate is rate-limiting, *e.g.* when bacteria are growing in two-liquid phase media, since the stimulation resulted from the rhamnolipid-enhanced uptake of these hydrophobic substrates.